

Traumatic Brain Injury Induces Central Insulin Resistance

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Abstract

TBIs produce a wide-range of neurological issues including, but not limited to, subdural hematoma, cognitive deficits, and in extreme cases, death. Although many mechanisms and consequences of TBI are well studied, there remains a gap in the understanding of the mechanisms by which repeated TBIs result in worse deficits than single injuries. Our lab has previously shown that repeated injuries occurring close in time impair the brain's metabolic recovery. In order to better understand the molecular mechanisms underlying the metabolic crisis associated with repeated TBI, we focused on central insulin signaling in mice injured once or twice, 24 hours apart. Insulin sensitivity assays were conducted by treating brain slices with insulin and measuring phosphorylation of Akt (protein kinase B) by Western Blot. Results showed that TBI induces brain insulin resistance by two days post-injury (DPI), which recovers by seven DPI in once-injured mice, but persisted until 28 DPI in mice that were injured twice. Further, obese (thus whole-body insulin resistant) mice that underwent TBI showed longer lasting reductions in Akt signaling. Further investigation of the mechanism by which brain tissue becomes insulin resistant may aid in the understanding and treatment of patients having undergone a TBI.

Introduction

Traumatic brain injuries—described by the CDC as a bump, blow, or jolt to the head—are a significant problem among athletes, members of the armed forces, as well as all civilians (CDC 2014). Each year in the US, an estimated 1.4 million TBIs occur. However, it is believed that there far more injuries than included in this statistic due to the injuries being treated outside of a physician's office or in military facilities in foreign

countries (Langlois *et al.*, 2006). A more severe problem arises when those who suffer an initial TBI suffer a second injury before the first resolves. In some cases, a second injury before the first has resolved has led to subdural hematoma, cognitive deficits, hemiparesis, and in extreme cases, death (Cantu & Gean, 2010). Moreover, following just one injury, the brain is characterized by hyperglycolysis, in which the energy demands are increased in order to meet the demands from the injury (Bergsneider *et al.*, 1997; Oddo *et al.*, 2008). And following a period of hyperglycolysis, the brain experiences an even longer period of hypoglycolysis (Prins *et al.*, 2013). These metabolic dysfunctions are associated with an increase in vulnerability, such that even a mild second injury may result in a worse outcome (Prins *et al.*, 2010, 2013; Fujita *et al.*, 2012; Weil *et al.*, 2014). Possible mechanisms for the metabolic abnormalities following injury include altered GLUT3 transporter expression (Hamlin *et al.*, 2001) and oxidative stress (Kammaksh *et al.*, 2014). However, it may be possible that a mediator of brain glucose utilization post-injury is insulin. Although insulin was thought to have no effect on glucose control in the brain (Oddo *et al.*, 2008), its local synthesis and transporter density in the blood brain barrier suggest that it may have an important effect (Banks, 2004; Ghasemi *et al.*, 2013).

Downstream of the insulin receptor are insulin receptor substrate proteins that couple the receptor to the phosphoinositide 3-kinase (PI3K) cascade (Gual *et al.*, 2005). Further downstream after PI3K has been activated, protein kinase B (Akt) is phosphorylated (Lima *et al.*, 2002). And although the effect of insulin on glucose utilization in the brain is not completely understood, it has been used as a therapeutic agent due to the increasing evidence for brain insulin-dependent glucose utilization (Gerozissis, 2008). Additionally, brain insulin resistance has been linked to

neurodegenerative diseases such as Parkinson's and Alzheimer's (Cardosa *et al.*, 2009). Perhaps most important for insulin's use as a therapeutic agent is its role in neurogenesis and reduction of neuroinflammation (Bateman & McNeill, 2006; Adzovic *et al.*, 2015). Further, obesity, and perhaps the insulin resistance seen with type 2 diabetes as a result of obesity, has been shown to increase mortality and complications after sustaining blunt trauma (Brown *et al.*, 2005). But since little is known about the relationship of TBI and brain insulin signaling, there remains a critical need to understand the biochemical mechanism behind the metabolic abnormalities to help improve outcome for future patients.

Materials and Methods

Animals

C57/Bl6 mice were obtained from Charles River and bred in our colony. Animals were maintained in a temperature and humidity controlled vivarium on a 14:10 light/dark cycle. Mice were given *ad-libitum* access to food (Teklad #8640, Harlan unless otherwise specified) and water. The study was conducted in accordance with The Ohio State University guidelines for the care and use of animals under protocols approved by the Institutional Animal Care and Use Committee.

Traumatic Brain Injury

At 6 weeks of age, male mice were subjected to either a mild TBI or sham procedure. Mice were anesthetized and their scalps shaved. They were then secured into a stereotaxic frame and skull surfaces were exposed. The impactor tip (round 3mm

diameter, Leica) was placed on the skull (-2 mm AP, -2 mm ML relative to bregma), retracted, and driven into the skull at 3 m/s to a depth of 1.4 mm. Anesthesia was turned off immediately prior to impact in order to measure the amount of time to regain consciousness. Sham procedures were identical except after the impactor was placed gently on the skull, it was then retracted. The return to consciousness—or time to awake (TTA)—was measured by placing each mouse on its back after suturing the scalp post-injury, and watching for the mouse to bring itself onto all four paws. Mice undergoing TBI had their skin closed immediately after injury, while still unconscious with sterile surgical clips, and sham mice had their skin clipped just before removal from anesthesia. Each mouse underwent two procedures 24 h apart—a manipulation that produced three groups: sham/sham, TBI/sham, or TBI/TBI. All animals were treated with 0.1 mg/kg subcutaneous buprenorphine and monitored for pain/discomfort throughout the study. Additional buprenorphine was available at 12 h intervals for these mice.

Diet Induced Obesity

Diet-induced obesity was achieved using a maternal obesity model (Samuelsson *et al.*, 2008), which developmentally programs hyperphagia. Additionally, the maternal diet-induced obesity model exposes pups to neonatal hyperinsulinemia. At 4 weeks of age, female mice were given either a low-fat standard chow diet (LFD) (10% of calories from fat) or a high-fat diet (HFD) (60% of calories from fat) (D12492, Research Diets). Mice were given *ad-libitum* access. Females were then paired with a breeder male at 6 weeks of age. Pregnant mothers maintained their respective diets through gestation and lactation. At 3 weeks of age, pups were weaned onto the same diets as their mothers.

Ex-vivo insulin stimulation

Mice were decapitated and fresh brains were immediately collected. The frontal lobe of the left hemisphere (injury side) was sliced at 0.4 mm intervals using a McIlwain tissue chopper. Slices were then maintained in oxygenated artificial cerebrospinal fluid (aCSF) [containing 127 nM NaCl, 24 nM NaHCO₃, 1.2 nM NaH₂PO₄, 2.5 nM KCl, 1nM MgCl, 25 nM glucose, 2 nM CaCl₂] at 37 °C for 20-30 min. Half of the slices from each left hemisphere were then incubated with 10 nM insulin (Sigma, St. Louis, MO, USA), and the other half with aCSF for 10 min at 37 °C. Following incubation, tissues were washed in ice-cold aCSF and lysed in radio-immunoprecipitation assay buffer containing phosphatase and protease inhibitors (Invitrogen). Total protein concentration was measured using a bicinchoninic acid protein assay (BCA; Bio-Rad).

Western Blotting

Protein analysis was performed by subjecting protein lysates to SDS-PAGE. 40 µg of protein was electrophoresed into a precast gel (Bio-Rad, 4-15%), transblotted onto a polyvinylidene difluoride membrane, and then blocked with 5% milk in tris-buffered saline (TBS). Following, membranes were incubated overnight at 4°C in TBS with 0.01% tween and rabbit anti-phosphorylated Akt (1:3,000 Ser 473 Cell Signaling) and GAPDH (1:2,000 Cell Signaling) antibodies, then goat anti-rabbit IgG HRP-conjugated secondary antibody (1:2,000 R & D) and visualized with enhanced chemiluminescent substrate (Invitrogen). Membranes were stripped (GM Biosciences) following this analysis, and reblotted with anti-rabbit pan Akt (1:2,000), Cell Signaling). The level of protein

expression was determined by densitometry calculation via Image J (Abràmoff *et al.*, 2004) and reported as pAkt(Ser473):pan Akt.

Histology Analysis

Phosphorylated Akt staining photomicrographs were captured at 10 x magnification throughout the sensorimotor cortex spanning bregma 0.02 to -1.34 mm (Paxinos & Franklin, 2004). Cell numbers were quantified in a 0.04 mm² area using ImageJ software and analyzed by cell number/mm². Data are collected from both hemispheres, but no significant differences were found. Thus, the data are reported as an average of the two.

Silver Staining

Axonal degeneration was assessed by silver staining using a NeuroSilver kit (FD Neurotechnologies) according to the manufacturer's instructions. Brain sections spanning the entire forebrain (roughly 12 section/brain) were analyzed at 10X magnification by a blinded observer. The level of silver staining was assessed on a four-point scale (0, no staining; 3, presence of silver staining in multiple white matter tracts throughout the forebrain).

Statistical Analysis

Statistical analysis was performed using SPSS Version 23 (IBM Corp., Armonk, NY, USA). Insulin stimulation data for time course experiments were analyzed via ANOVA (injury x insulin dose). Diet experiments with insulin stimulation data were also analyzed

via ANOVA (injury x insulin dose x diet). Qualitative axonal degeneration data were analyzed via the Mann-Whitney *U*-test.

Experimental Protocol

Experiment 1:

Traumatic brain injury effects on brain insulin resistance

Male mice underwent one TBI followed by a sham procedure (24 h apart), two TBIs (24 h apart), or two sham procedures (24 h apart), and brain tissue was collected for *ex-vivo* insulin stimulation on two (sham, $n = 6$, one injury, $n = 7$; two injuries, $n = 7$), seven (one injury, $n = 7$; two injuries, $n = 6$), 14 (one injury, $n = 5$; two injuries, $n = 6$), or 28 (two injuries, $n = 8$) days post-injury (Fig. 1A).

Experiment 2:

Diet-induced obesity effects on traumatic brain injury

Male mice that were weaned onto their mother's diet underwent two TBIs (24 h apart) or two sham procedures (24 h apart). Thus, we were given four groups: LFD/sham, LFD/TBI, HFD/sham, HFD/TBI. Brain tissue was collected for *ex-vivo* insulin stimulation two days post-injury ($n = 10$ /group, total $n = 40$).

Results

Traumatic Brain Injury Induces Long-Lasting Insulin Resistance

To determine brain insulin receptor sensitivity as a result of TBI, brains from sham, once, and twice injured mice were treated *ex-vivo* with 0 or 10 nM insulin. Phosphorylation of

Akt at serine 473 relative to total Akt was used as an indicator of insulin receptor activation (Fig. 2A). Western blot analysis showed a significant increase in Akt phosphorylation in sham brains when treated with insulin ($F_{2,14} = 4.436, P = 0.036$), but no insulin-dependent increase in Akt phosphorylation in injured mice at two days post-injury (DPI) (Fig. 2B). At seven DPI brain tissue from mice having undergone one injury recovered insulin sensitivity ($F_{1,8} = 8.377, P = 0.023$), which persisted at 14 DPI ($F_{1,5} = 9.110, P = 0.039$). However, mice injured twice remained insulin resistant at both 7 and 14 DPI (Figs. 2C,D). Because insulin sensitivity persisted in the once-injured animals at 14 DPI (Fig 2D), only the twice-injured animals were repeated until 28 DPI. At this time point, insulin sensitivity assessed through the ratio of phosphorylated Akt:pan Akt was significantly increased post insulin treatment ($F_{1,11} = 6.294, P = 0.0310$; Fig. 2E).

High-fat diet has no effect on severity of injury

By using a maternal diet-induced obesity model, we were able to determine whether or not pre-existing, whole body insulin resistance exacerbates TBI-induced insulin receptor insensitivity. Pregnant females and their offspring were fed either a LFD or HFD, and at six weeks of age, the offspring underwent either a sham surgery or TBI. Mice fed the HFD had a significantly increased body mass ($F_{1,31} = 67.402, P < 0.0001$), but this did not have a noticeable effect on brain damage following TBI, as both HFD and LFD TBI animals showed no significant difference in TTA following injury (Fig. 3). Additionally, axonal degeneration assessed through silver staining showed that TBI increased axon damage relative to sham ($U = 1.5, P < 0.001$), but did so to a similar extent in both HFD

and LFD groups (Fig. 3). Thus, we can conclude that the effect of diet on insulin sensitivity appeared to be independent of severity of injury.

High-fat diet acutely increases traumatic brain injury-induced Akt phosphorylation

Ex-vivo insulin stimulation on HFD and LFD mice showed that brain tissue from LFD sham animals responded to insulin through a significant increase in phosphorylated Akt expression ($F_{1,11} = 6.101$, $P = 0.033$), whereas brain tissue from the HFD sham mice did not increase expression of phosphorylated Akt post-insulin stimulation. Further, both HFD and LFD TBI animals experienced insulin resistance following injury. However, although insulin-dependent Akt phosphorylation did not increase relative to control after injury, phosphorylated Akt expression was significantly greater in HFD-TBI mice when compared to HFD-sham mice ($F_{1,28} = 5.003$, $P = 0.033$). Thus, the data suggest that TBI influenced an increase in insulin-independent Akt phosphorylation in HFD mice.

Additionally, the insulin-independent increase in expression of phosphorylated Akt was significantly higher in HFD mice than LFD mice following TBI ($F_{1,33} = 4.806$, $P = 0.036$). When taken together, the data suggest that at two DPI, HFD increased Akt phosphorylation post-TBI (Fig. 4A and B).

Discussion

Our experiment design was based on two main findings: following TBI, insulin resistance has been shown to increase mortality (Ley *et al.*, 2011), and the brain has a period of disrupted metabolism (Prins *et al.*, 2013, Weil *et al.*, 2014). The results showed that in

mice, insulin resistance following one mild TBI persists until 7 DPI, but persists until 28 DPI following two injuries.

It may be possible that the reduced phosphorylation of Akt plays a role in the exacerbated outcomes following TBI. Akt has been shown to have a neuroprotective effect (Zhao *et al.*, 2006), and also when inactivated can exacerbate damage from injury (Miyawaki *et al.*, 2009). Our model shows that insulin was unable to activate /phosphorylate Akt. Although whether or not insulin directly influences brain glucose metabolism remains under debate (Oddo *et al.*, 2008), increasing evidence shows a connection to insulin-dependent glucose homeostasis (Gerozissis, 2008). Further, insulin activity has been shown to stimulate the uptake and utilization of glucose following oxidative stress (Duarte, *et al.*, 2006), which is a component of repeated TBI (Kammaksh, *et al.*, 2016). We are unaware of any other report on the changes seen in insulin-dependent Akt phosphorylation following TBI. Our data make sense due to the many other studies that have shown vulnerability of the brain after receiving a TBI. And further, it may be possible that the exacerbated damage seen following repeated TBIs occurring close in time may be impacted by brain insulin resistance.

In order to further explore the effect of insulin resistance and TBI, we used a diet-induced obesity model—hoping to create brain insulin resistance. Mothers of mice and their offspring were fed either a HFD or LFD and subjected to either a sham procedure or injury. Consistent with a previous report (Arnold *et al.*, 2014), the data showed that the diet-induced obesity induced insulin resistance. We were then able to examine the effects of prior whole-body insulin resistance on the outcome of injury and recovery following TBI. Results showed that although the phosphorylation of Akt was significantly higher in

HFD-TBI mice compared to the LFD-TBI group. Further, the ratios of phosphorylated Akt:pan Akt were higher in insulin-stimulated (10 nM insulin in artificial cerebrospinal fluid) and control (artificial cerebrospinal fluid only) HFD mice following TBI, suggesting an insulin-independent increase in Akt phosphorylation. Interestingly, exogenous insulin did not increase the phosphorylation of Akt. Additionally, Akt phosphorylation can happen through various signaling pathways, thus changes in the amount of phosphorylated Akt may have been a result of other biochemical processes.

Although we discovered insulin resistance in our animal models, the cause of the mechanism of insulin resistance remains unknown. Inflammation has been shown to be associated with insulin resistance (Dandona *et al.*, 2004), obesity (Hotamisligil, 2006), and TBI (Werner and Engelhard, 2007). Thus, the role of inflammation may have contributed to, or possibly be a mechanism for the associated insulin resistance that resulted. Further investigation of the post-TBI mechanism by which inflammatory molecules may affect neuronal metabolism and recovery may allow for better treatment to decrease the resulting deficits.

Figures

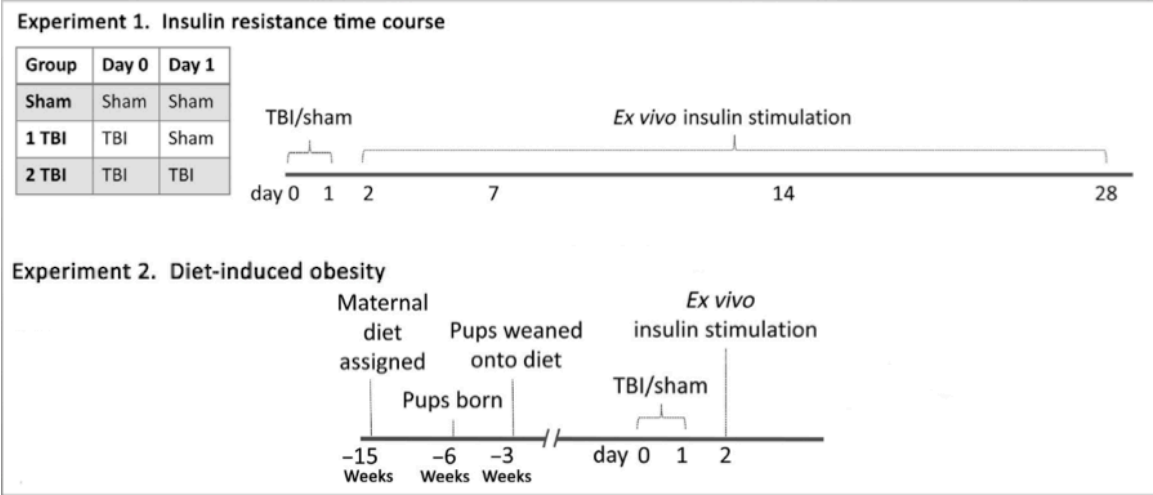


Figure 1: Experimental timeline. Experiment 1: Insulin resistance time course. A time course of insulin receptor sensitivity was assessed in groups of sham, one injury, and two injuries on days 2, 7, 14, and 28 post injury. Experiment 2: Diet-induced obesity. Mice raised on a maternal obesity model (HFD) or standard diet (LFD) underwent sham or TBI procedures 24 h apart. Their insulin sensitivity was tested two DPI and tissue was stained following sacrifice 16 DPI.

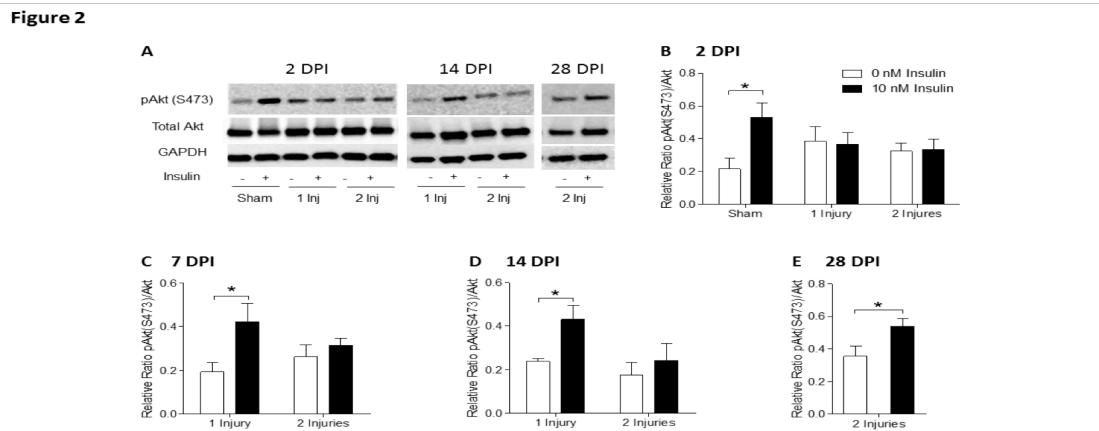


Figure 2: TBI-induced brain insulin resistance. Western blots of frontal brain tissue from sham (sham/sham), 1 injury (TBI/sham), and 2 injuries (TBI/TBI) mice with (+) or without (-) insulin stimulation. Tissue was collected 2, 7, 14, or 28 days post injury (DPI). Analysis shows that insulin stimulation leads to activation of Akt in sham injured, two but not in injured mice two DPI. Mice that were injured a single time recover insulin sensitivity by 7 DPI, which continues 14 DPI. Mice injured twice remained insulin resistant through 14 DPI, and insulin sensitivity was normalized by 28 DPI.

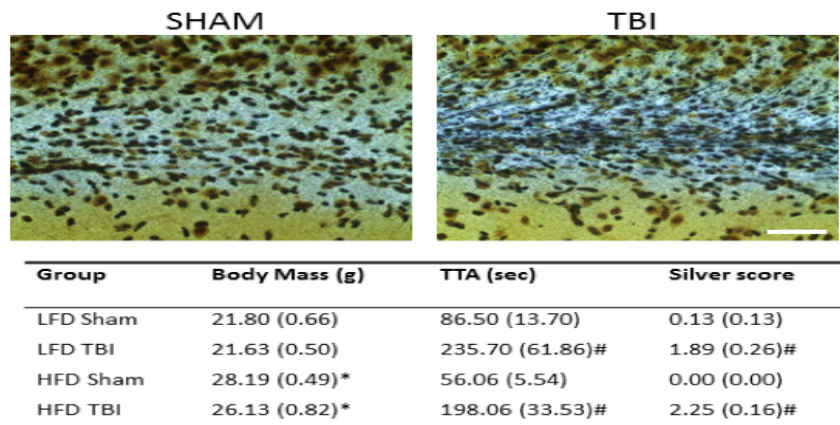


Figure 3: High fat diet effects on insulin sensitivity are independent of injury severity. Axonal degeneration was assessed by silver staining mice brain tissue. Silver score analysis (0 = no staining, 3 = presence of silver staining in multiple white matter tracts throughout the forebrain) shows that TBI significantly increased axon damage relative to sham, but was not affected by diet. Further, injury also shows a significant difference in relation to sham time to awake (TTA), but is independent of diet.

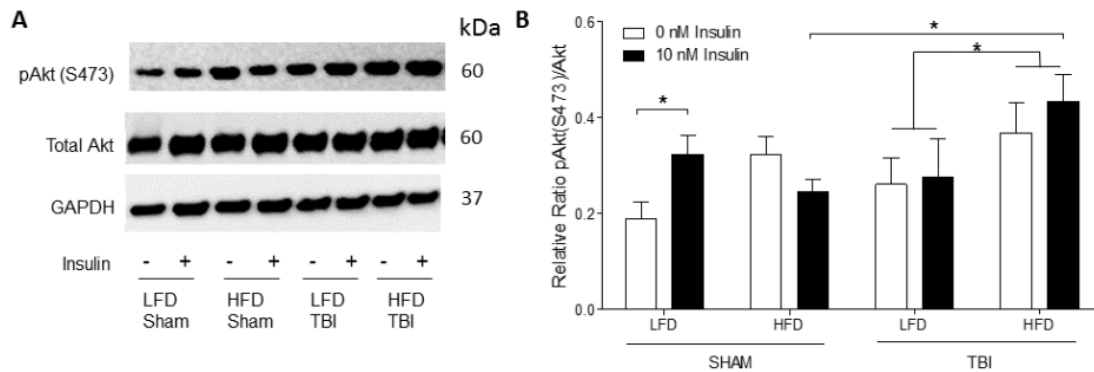


Figure 4: High fat diet induces central insulin resistance and, with TBI, increased Akt phosphorylation. Western blots of frontal brain tissue from low-fat diet (LFD) and high-fat diet (HFD) sham or TBI mice (+) or without (-) insulin stimulation. Analysis shows that brain tissue from sham animals fed a LFD responds with an increase in pAkt expression, and tissue from HFD-fed animals does not. Both LFD- and HFD-fed mice experience brain insulin resistance following a TBI. Brain tissue from HFD-TBI mice exhibits an increase in Akt phosphorylation compared to HFD-sham mice brain tissue, suggesting that TBI increases insulin-independent Akt phosphorylation in HFD-fed mice.

Acknowledgements

I want to thank Dr. Zachary Weil and Dr. Ekaterina Karelina for their support, teaching, and volunteered time throughout the entire process. Additionally, I want to thank Dr. Brian Focht for serving as a member of the thesis committee, and helping oversee the project. This work would not be possible without the selfless attitudes of these individuals.

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